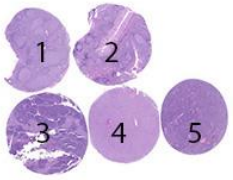
Assessment Run 52 2018

Terminal deoxynucleotidyl Transferase (TdT)

Material
The slide to be stained for TdT comprised:



1-2. Tonsil, 3. Thymus, 4-5. Thymoma

All tissues were fixed in 10% neutral buffered formalin.

Criteria for assessing TdT staining as optimal included:

- A strong, distinct nuclear staining reaction of dispersed "immature/pre-mature T-lymphocytes" in the interfollicular zones of the two tonsils[#].
- An at least moderate, distinct nuclear staining reaction of virtually all cortical thymocytes of the normal thymus.
- An at least weak to moderate, distinct nuclear staining reaction of the vast majority of immature T-cells intermingling with the neoplastic cells of the thymoma (tissue core no. 4).
- A strong, distinct nuclear staining reaction of virtually all immature T-cells intermingling with neoplastic cells of the thymoma (tissue core no. 5).
- No nuclear staining reaction of mature T- and B-cells in the tonsils and the vast majority of medullary thymocytes of the normal thymus.

[#]J Clin Invest. 2012;122(4):1403–1415.

Participation

Number of laboratories registered for TdT, run 52	238
Number of laboratories returning slides	225 (95%)

Results
225 laboratories participated in this assessment. 184 (82%) of these achieved a sufficient mark (optimal or good). Table 1 summarizes antibodies (Abs) used and assessment marks (see page 2).

The most frequent causes of insufficient staining were:
- Inefficient HIER (too short heating time or use of non-alkaline buffer)
- Too low concentration of the primary antibody
- Less successful antibodies (numerous pAbs from various manufacturers)
- Use of less sensitive detection systems
- Unexplained technical issues

Performance history
This was the third NordiQC assessment of TdT. The overall pass rate increased compared to previous run 40 (see Table 2).

Table 2. **Proportion of sufficient results for TdT in the three NordiQC runs performed**

	Run 18 2006	Run 40 2014	Run 52 2018
Participants, n=	62	185	225
Sufficient results	93%	78%	82%

Conclusion
The mAb clone **SEN28** and the rmAb clone **EP266** were in this assessment the most robust and successful antibodies for TdT. Efficient HIER, preferable in an alkaline buffer in combination with a sensitive and specific 3-step polymer/multimer based detection system gave the highest proportion of optimal results. For both primary Abs, optimal results could be obtained on the 4 main IHC systems - Omnis (Dako), Autostainer (Dako), Bond (Leica) and BenchMark (Ventana). The Ready-to-use systems PA0339 (Leica) and IR093 (Dako) based on mAb SEN28 and rmAb EP266, respectively, provided a higher proportion of optimal results than the laboratory modified protocol settings, typically prolonging incubation time of the primary Ab (PA033) or applying a more sensitive detection system (Envision Flex+ (IR093)). Only 2% (1 of 58) of the laboratories using a pAb could produce an optimal result.
Thymus is recommended as positive tissue control for TdT. Virtually all cortical thymocytes must show an

at least moderate and distinct nuclear staining reaction. Tonsil can be used as negative tissue control, no staining reaction should be seen in mature B-cells (e.g. the mantle zone and germinal centre B-cells).

Table 1. **Antibodies and assessment marks for TdT, run 52**

Concentrated antibodies	n	Vendor	Optimal	Good	Borderline	Poor	Suff.[1]	Suff. OPS[2]
mAb clone **SEN28**	66 3 2 1 1	Leica/Novocastra Diagnostic Biosystems Thermo/NeoMarkers Monosan Immunologic	20	29	19	5	67%	70%
rmAb clone **EP266**	13 2 1 1	Agilent/Dako Cell Marque Diagnostic Biosystems Epitomics	11	3	3	0	82%	87%
pAb **A3524**[3]	2	Agilent/Dako	0	1	1	0	-	-
pAb **ILP 0049**	3	Immunologic	0	1	2	0	-	-
pAb **338A-76**	2	Cell Marque	0	0	1	1	-	-
pAb **CP134**	1	Biocare Medical	0	1	0	0	-	-
pAb **44811**	1	Menarini Diagnostics	0	1	0	0	-	-
Ready-To-Use antibodies								
mAb clone **SEN28 PA0339**	11	Leica/Novocastra	6	5	0	0	100%	100%
mAb clone **SEN28 PA0339**[4]	5	Leica/Novocastra	2	1	1	1	-	-
mAb clone **SEN28 8243-C010**	1	Sakura FineTek	1	0	0	0	-	-
mAb clone **SEN28 MAB-0197**	1	Maixin	1	0	0	0	-	-
mAb clone **SEN28 MS-1105-R7**	1	Thermo/Neomarkers	0	1	0	0		
rmAb clone **EP266 IR093**	36	Agilent/Dako	26	8	2	0	94%	95%
rmAb clone **EP266 IR093**[4]	17	Agilent/Dako	17	0	0	0	100%	100%
rmAb clone **EP266 MAD-000659QD**	2	Master Diagnostica	1	1	0	0	-	-
rmAb clone **EP266 338R-28**	1	Cell Marque	1	0	0	0	-	-
rmAb clone **EP266 Unknown**	1	Unknown	0	1	0	0		
pAb **760-2670**	45	Ventana/Cell Marque	1	39	4	1	89%	100%
pAb **338A-78**	4	Cell Marque	0	4	0	0	-	-
pAb **IR001**[3]	1	Agilent/Dako	0	1	0	0	-	-
Total	225		87	97	33	8	-	
Proportion			39%	43%	15%	3%	82%	

1) Proportion of sufficient stains (optimal or good).
2) Proportion of sufficient stains with optimal protocol settings only, see below.
3) Product discontinued.
4) Ready-to-use product developed for a specific semi/fully automated platform by a given manufacturer but inappropriately applied by laboratories on other non-validated semi/fully automatic systems or used manually.

Detailed analysis of TdT, Run 52

The following protocol parameters were central to obtain optimal staining:

Concentrated antbodies

mAb clone **SEN28**: Protocols with optimal results were all based on HIER using Target Retrieval Solution (TRS) pH 9 (3-in-1) (Dako) (5/8)*, Cell Conditioning 1 (CC1; Ventana) (11/42), Bond Epitope Retrieval Solution 2 (BERS2; Leica) (3/14) or Borg Decloaker (Biocare Medical) (1/1) as retrieval buffer. The mAb was typically diluted in the range of 1:25-1:50 depending on the total sensitivity of the protocol employed.

Using these protocol settings, 30 of 43 (70%) laboratories produced a sufficient staining reaction (optimal or good).
* (number of optimal results/number of laboratories using this buffer)

rmAb clone **EP266**: Protocols with optimal results were typically based on HIER using TRS pH 9 (3-in-1) (Dako) (3/5), CC1 (Ventana) (5/8), BERS2 (Leica) (1/1) or DBS Montage EDTA solution (Diagnostic Biosystems) (1/1) as retrieval buffer. The mAb was typically diluted in the range of 1:10-1:100 depending on the total sensitivity of the protocol employed. Using these protocol settings, 13 of 15 (95%) laboratories produced a sufficient staining reaction (optimal or good). One laboratory produced an optimal result without any pre-treatment.

Table 3. **Proportion of optimal results for TdT for the most commonly used antibodies as concentrate on the 4 main IHC systems***

Concentrated antibodies	**Dako Autostainer Link / Classic**		**Dako Omnis**		**Ventana BenchMark XT / Ultra**		**Leica Bond III / Max**	
	TRS pH 9.0	TRS pH 6.1	TRS pH 9.0	TRS pH 6.1	CC1 pH 8.5	CC2 pH 6.0	ER2 pH 9.0	ER1 pH 6.0
mAb clone **SEN28**	3/3**	-	2/4	-	8/30 (27%)	-	2/5 (40%)	0/2
rmAb clone **EP266**	1/3	-	2/2	-	5/8 (63%)	-	1/1	-

* Antibody concentration applied as listed above, HIER buffers and detection kits used as provided by the vendors of the respective systems.
** (number of optimal results/number of laboratories using this buffer)

Ready-To-Use antibodies and corresponding systems
mAb clone **SEN28**, product no. **PA0339**, Leica, Bond III/MAX:
Protocols with optimal results were typically based on HIER in using BERS2 (efficient heating time 10-20 min. at 93-97°C), 15-20 min. incubation of the primary Ab and Bond Refined (DS9800) as detection system. Using these protocol settings, 7 of 7 (100%) laboratories produced a sufficient staining result.

rmAb clone **EP266**, product no. **IR093**, Dako, Autostainer+/Autostainer Link:
Protocols with optimal results were typically based on HIER in PT-Link using TRS pH 9 (3-in-1) (efficient heating time 10-20 min. at 95-97°C), 20-30 min. incubation of the primary Ab and EnVision FLEX/FLEX+ (K8000/K8002) as detection systems. Using these protocol settings, 18 of 19 (95%) laboratories produced a sufficient staining result.

pAb, product no. **760-2670**, Ventana, BenchMark XT, GX, ULTRA:
Protocol with optimal result was based on HIER using CC1 (efficient heating time 48 min. at 100°C), 24 min. incubation of the primary Ab and OptiView (760-700) as detection system. Using these protocol settings, 1 of 1 (100%) laboratory produced a sufficient (optimal) staining result.

Table 4 summarizes the proportion of sufficient and optimal marks for the most commonly used RTU systems. The performance was evaluated both as "true" plug-and-play systems performed strictly accordingly to the vendor recommendations and by laboratory modified systems changing basal protocol settings. Only protocols performed on the specific IHC stainer device are included.

Table 4. **Proportion of sufficient and optimal results for TdT for the most commonly used RTU IHC systems**

RTU systems	**Recommended protocol settings***		**Laboratory modified protocol settings****	
	Sufficient	Optimal	Sufficient	Optimal
Leica BOND MAX/III mAb SEN28 **PA0339**	100% (3/3)	0% (0/3)	100% (8/8)	75% (6/8)
Dako AS mAb EP266 **IR093**	92% (11/12)	50% (6/12)	100% (20/20)	90% (18/20)
VMS Ultra/XT/GX pAb **760-2670**	0% (0/1)	0%(0/1)	89% (34/38)	3% (1/38)

* Protocol settings recommended by vendor – Retrieval method and duration, Ab incubation times, detection kit, IHC stainer/equipment.
** Significant modifications: retrieval method, retrieval duration and Ab incubation time altered >25%, detection kit – only protocols performed on the specified vendor IHC stainer were included.

Comments

In this NordiQC assessments for TdT, the prevalent feature of an insufficient staining result was a too weak or completely false negative staining reaction of cells and structures expected to be demonstrated. This pattern was observed in 88% of the insufficient results (36 of 41). The remaining insufficient results were characterized by poor signal-to-noise ratio and/or false positive staining reaction compromising interpretation of the specific reactions.
Virtually all participating laboratories were able to stain TdT in cells with high-level expression as immature T-cells in the tonsils and immature T-cells in the thymoma (tissue core no. 5), whereas demonstration of TdT in cortical thymocytes of the thymus and immature T-cells in the thymoma (tissue core no. 4) was more challenging and could only be demonstrated when appropriate protocol settings were applied.

The mAb clone SEN28 was the most widely used antibody for demonstration of TdT (see Table 1). Used as a concentrate within a laboratory developed (LD) assay, the mAb clone SEN28 gave an overall pass rate of 67% (49 of 73). Optimal results could be obtained on all four main IHC platforms from Dako, Leica and Ventana (see Table 3). The prevalent protocol settings for optimal performance was use of efficient HIER in an alkaline buffer and a relatively high concentration of the primary Ab (average working dilution of 1:35, range 1:25-to 1:100). There was no significant difference in performance between the use of a 3-step multimer/polymer (e.g. OptiView+/- amplification, Bond Refine or Flex+) or a 2-step multimer/polymer (e.g. UltraView or Flex) detection system. Protocols giving an insufficient result, applied protocol settings similar to laboratories obtaining an optimal result. Therefore, it is difficult to elucidate on the technical problems and give helpful advises to the participants. However, it has been noticed in a NordiQC reference laboratory, that the mAb clone SEN28 may benefit from dilution in low pH diluent (pH 6.0-6.2, e.g. Renoir Red from Biocare Medical).

The rmAb EP266 used within a LD-assay gave an overall pass rate of 82% (14 of 17) of which 65% (11 of 17) were assessed as optimal. Protocols based on the rmAb EP266 stained endothelial structures with a distinct cytoplasmic staining reaction. This aberrant staining pattern did not interfere with the specific reaction for TdT and was accepted in this assessment. Protocols assessed as optimal were typically based on HIER in alkaline buffer, high concentration of the primary Ab (average working dilution of 1:41, range 1:10 to 1:100) and use of a sensitive 3-step multimer/polymer detection system (e.g. OptiView or Flex+). All protocols (3 of 3) giving an insufficient mark, used a low sensitive 2-step multimer/polymer detection system (e.g UltraView or Flex).

56% (126 of 225) of the laboratories used a Ready-To-Use (RTU) system for TdT.
In this assessment, the RTU system PA0339 based on mAb clone SEN28 (Leica) was the most successful assay for demonstration of TdT and provided a pass rate of 100% (11 of 11) of which 55 % (6 of 11) were optimal. However, optimal results could only be obtained using laboratory modified protocol settings (see Table 4), typically prolonging incubation time of the primary Ab.

The RTU system IR093 based on rmAb clone EP266 (Dako) also provided a high proportion of sufficient results (94%, 34 of 36) and optimal results (72%, 26 of 36). Both vendor recommended, and laboratory modified protocol setting could be used to obtain an optimal result (see Table 4), but proportion of optimal results was significant higher applying laboratory modified protocol settings, typically substituting Envision Flex with Envision Flex+.
Surprisingly, using the RTU system IR093 "off-label" (used in a manner not fulfilling intended use – e.g. another automatic platform than it was developed for), all protocols (17 of 17) were assessed as optimal. The RTU system was used by 88% (15 of 17) of the participants on the Omnis and protocol settings were similar to settings as described above. This indicate that the rmAb clone EP266 is rather robust and resistant to changes in protocol settings. In general, it cannot be recommended to use a RTU outside the system and platform is was developed for.

The Ventana RTU system for the BenchMark IHC platform based on pAb 760-2670 provided an overall pass rate of 89% (40 of 45) but only 2% (1 of 45) were assessed as optimal. As mentioned in the report Run 40, the pAb 760-2670 RTU system had a tendency to produce background staining. In this assessment, the same pattern was seen. The staining reaction was typically displayed as a weak aberrant cytoplasmic staining reaction of germinal centre B-cells and squamous epithelial cells of the tonsils. This weak cytoplasmic staining reaction did not affect the evaluation and was therefore accepted as long as interpretation of the specific nuclear TdT expression was not compromised. In the last two runs, the RTU system 760-2670 could only produce 2% (2 of 82) optimal result. Thus, and from a technical point of view, this assay seems challenging and it may be advisable to change to a concentrated format of a more robust clone (e.g. EP266 and SEN28) and recalibrate protocol settings. In this assessment, the vast majority of laboratories modified their protocol settings (see Table 4).

This was the third assessment of TdT in NordiQC (see Table 2). A pass rate of 82% was obtained, which is an improvement compared to 78% in run 40, 2014. For laboratories participating for the first time (n=82), the pass rate was 78%. This was marginally lower compared to labs that also participated in run 40 (n=143), obtaining an overall pass rate of 84%. The extended use of robust monoclonal antibodies (mAb SEN28 and rmAb EP266) both within LD-assays but also as RTU systems, accounted for the overall improvement of the pass rate. In this assessment, protocols based on pAbs provided a significantly lower proportion of optimal results compared to protocols based on monoclonal antibodies. Grouped together, both LD-assays and RTU systems, only 2% (1 of 58) of the laboratories applying a pAb could produce an optimal result, whereas 53% (88 of 167) of the laboratories using either mAb SEN28 or rmAB EP266 produced an optimal staining result.

Controls

Thymus is recommended as control for detection of TdT. The protocol must be calibrated to give an intense nuclear staining reaction in virtually all cortical thymocytes. Scattered thymocytes situated in medulla will be positive. In tonsil, dispersed pre-mature T-cells localised to the interfollicular zones will display a strong distinct nuclear staining reaction. This reaction pattern was less reliable as positive control for TdT, as a false negative staining reaction of the thymoma (tissue core no. 4) could be seen. Mature B-cells of the mantle zone and germinal centre B-cells should be negative.

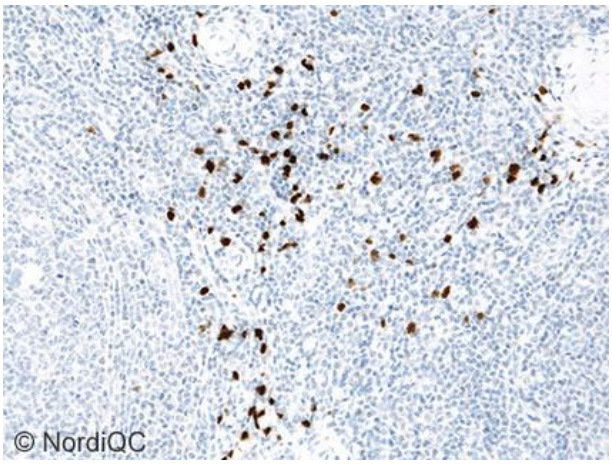


Fig. 1a (x200)
Optimal TdT staining of tonsil using the mAb clone SEN28, optimally calibrated, HIER in TRS (3-1) pH 9 (Dako) and a 3-step polymer based detection system (Flex+/Dako).
Dispersed pre-mature T-cells of the interfollicular zones show a strong and distinct nuclear staining reaction. Same protocol used in Figs. 2a - 4a.

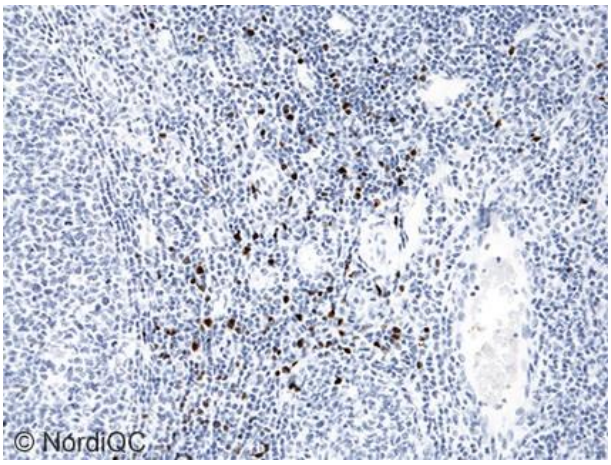


Fig. 1b (x200)
Insufficient staining of TdT in the tonsil using the mAb clone SEN28, too diluted and applying the less sensitive detection system Flex (Dako) – same field as in Fig. 1a. Although the pre-mature T-cells of the interfollicular zones display a relative strong nuclear staining intensity, the protocol provided too low sensitivity (compare Figs. 1a - 4b). Same protocol used in Figs. 2b - 4b.

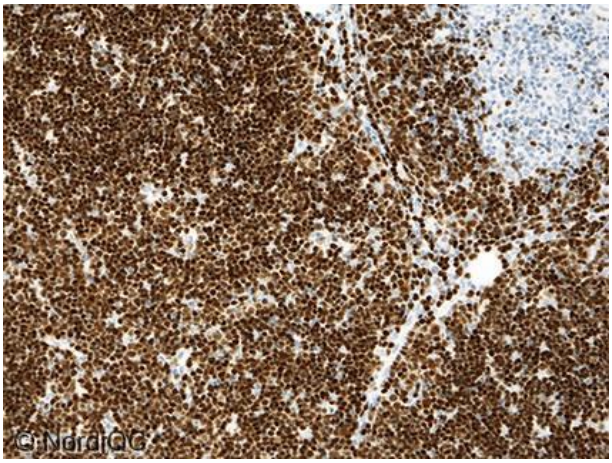


Fig. 2a (x200)
Optimal staining of TdT in the thymus using same protocol as in Fig. 1a. Immature cortical thymocytes and scattered pre-mature T-cells of medulla show a strong and distinct nuclear staining reaction.

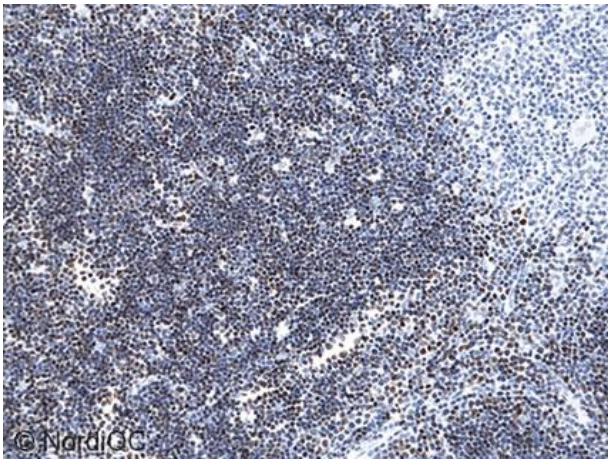


Fig. 2b (x200)
Insufficient staining of TdT in the thymus using same protocol as in Fig. 1b – same field as in Fig. 2a.
The staining intensity and proportion of positive cortical thymocytes is significantly reduced.

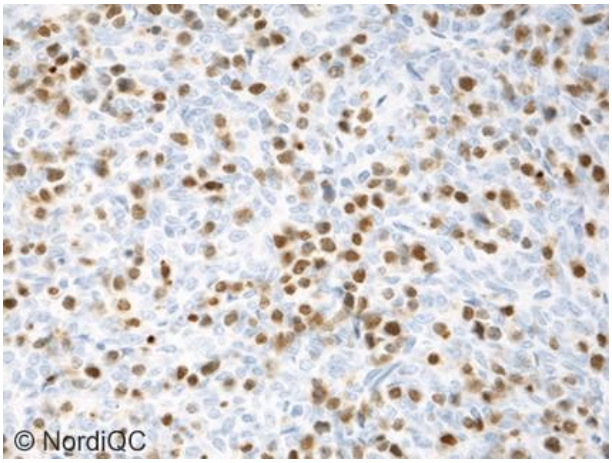


Fig. 3a (x400)
Optimal TdT staining of the thymoma (tissue core no. 4) using same protocol as in Figs. 1a and 2a. The vast majority of immature T-cells intermingling between the neoplastic cells show a weak to moderate but distinct nuclear staining reaction.

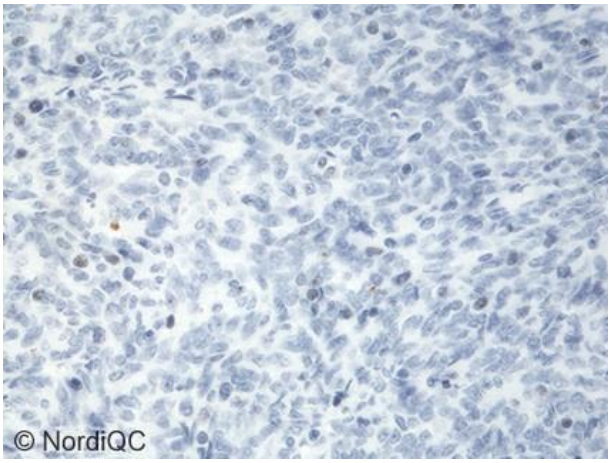


Fig. 3b (x400)
Insufficient TdT staining of the thymoma (tissue core no. 4) using same protocol as in Figs. 1b and 2b - same field as in Fig. 3a. The immature T-cells intermingling between the neoplastic cells are false negative or only faintly demonstrated in a small fraction of the total population of T-cells.

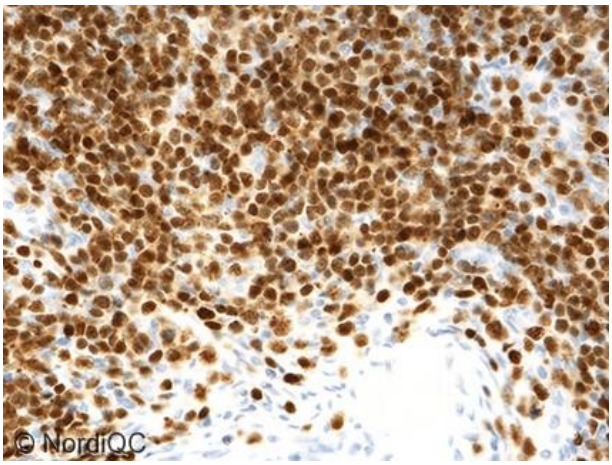


Fig. 4a (x400)
Optimal TdT staining of the thymoma (tissue core no. 5) using same protocol as in Figs. 1a – 3a. Virtually all the immature T-cells show a strong and distinct nuclear staining reaction.

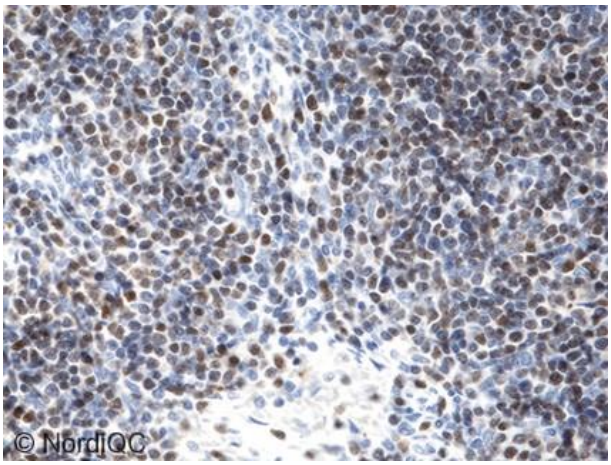


Fig. 4b (x400)
Insufficient TdT staining of the thymoma (tissue core no. 5) using same protocol as in Figs. 1b and 3b - same field as in Fig. 4a. The staining intensity of the immature T-cells is significantly reduced.

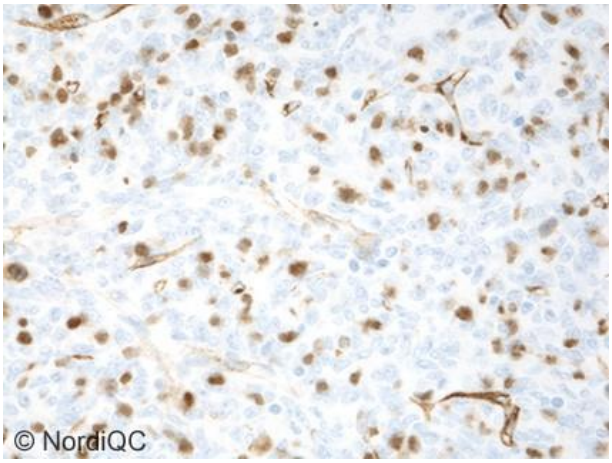


Fig. 5a (x400)
Optimal TdT staining of the thymoma (tissue core no. 4). The protocol was based on the rmAb clone EP266 as RTU format (IR093/Dako), HIER in TRS (3-in-1) pH 9 (Dako) and a 2-step polymer based detection system (Flex/Dako). The immature T-cells show the expected reaction pattern (see Fig. 3a), but endothelial cells display an aberrant cytoplasmic staining reaction. This aberrant staining pattern did not interfere with the specific reaction for TdT and was therefore accepted in this assessment.

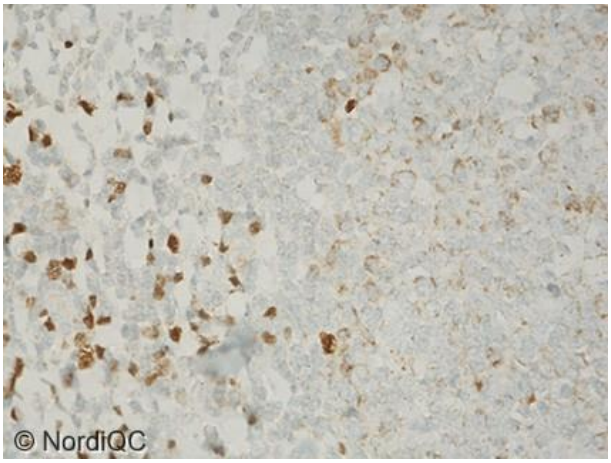


Fig. 5b (x400)
Insufficient TdT staining of the tonsil using a protocol providing too low sensitivity and poor signal-to-noise ratio. The protocol was based on the RTU format (760-2670/Ventana), HIER in CC1 (Ventana) and a 2-step multimer based detection system (UltraView/Ventana). The pre-mature T-cells in the interfollicular zone display too weak staining intensity (compare with Fig. 1a) and the mature germinal centre B-cells show an aberrant granular cytoplasmic staining reaction.

MB/LE/MV/RR 27.03.2018